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14. ABSTRACT
The main goal of the study funded by this grant was to test a hypothesis that cell fusion between tumor cells, or between tumor and normal cells, contributes to metastasis. This hypothesis suggests that cell fusion contributes to carcinogenesis through two mechanisms: by generating cells with diverse genetic and epigenetic properties and by providing tumor cells with qualities of normal cells that are required to travel throughout the body while remaining alive. This hypothesis can explain why tumor cells can grow at distant sites, why they express proteins that are normally expressed by cells of the metastasized tissue, and why only a minute fraction of cells released by primary tumors form metastases. The proposed research was designed to explain gene transfer between two lines of human prostate cancer cells PC3 in vivo and to determine whether this gene transfer is mediated by cell fusion and whether cell fusion affects metastatic potential of these cells. We found that gene transfer was mediated by a xenotropic mouse virus and developed approaches to generate cell hybrids for testing their metastatic potential

15. SUBJECT TERMS
Prostate cancer, cell fusion, metastasis, viruses, XMRV

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INTRODUCTION

The main goal of this study was to test a hypothesis that cell fusion between tumor cells, or between tumor and normal cells contributes to metastasis. This hypothesis suggests that cell fusion contributes to carcinogenesis through two mechanisms: by generating cells with diverse genetic and epigenetic properties, and by providing tumor cells with qualities of normal cells that are required to travel throughout the body while remaining alive. This hypothesis can also explain why cells tumor cells can grow at distant sites, why they express proteins that are normally expressed by cells of the metastasized tissue, and why only a minute fraction of cells released by the primary tumors form metastases.

BODY

This grant application was based on an observation made by Dr. Glinsky (Ordway Research Institute, Albany), who became a co-investigator and then a collaborator on this grant. Dr. Glinsky's laboratory reported (Glinsky et al., 2006) that if human prostate cancer cells PC3 expressing green fluorescent protein EGFP ("green" PC3 cells) or red fluorescent protein RFP ("red" PC3 cells) were injected into mice together the resulting tumors were often composed of cells that expressed both protein ("yellow" cells). The "yellow" cells had enhanced metastatic potential, which suggested that the horizontal gene transfer of the genetic information affected cell malignancy. We proposed to identify the mechanism of this transfer (Aim 1), with the main hypothesis being that the gene exchange was mediated by cell fusion, and to test whether cell fusion caused by viruses can affect ability of PC3 cells to metastasize (Aim 2).

The outcome of exploring Aim 1: To identify the mechanism of genetic exchange among prostate cancer cells. We proposed three hypotheses to explain how the genes encoding the fluorescent markers were transferred. Our favorite was that the transfer was caused by cell fusion, while the second idea was that the genes were exchanged through the engulfment of apoptotic bodies. The third possibility that we considered was that the mechanism of gene transfer is new or unanticipated, which is what we have found.

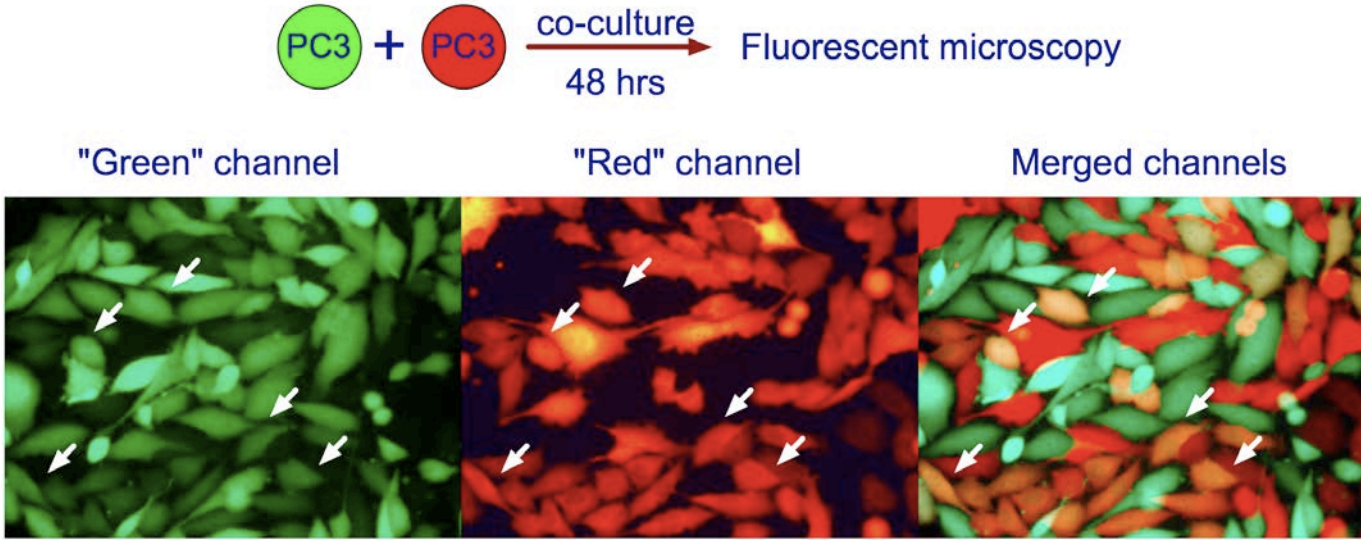


Figure 1. Prostate cancer PC3 cells exchange genetic markers in tissue culture. PC3 cells expressing EGFP ("green" cells) or RFP ("red" cells) were cultured together as indicated and analyzed by fluorescence microscopy. Some of the numerous cells that expressed both proteins are indicated with arrows.

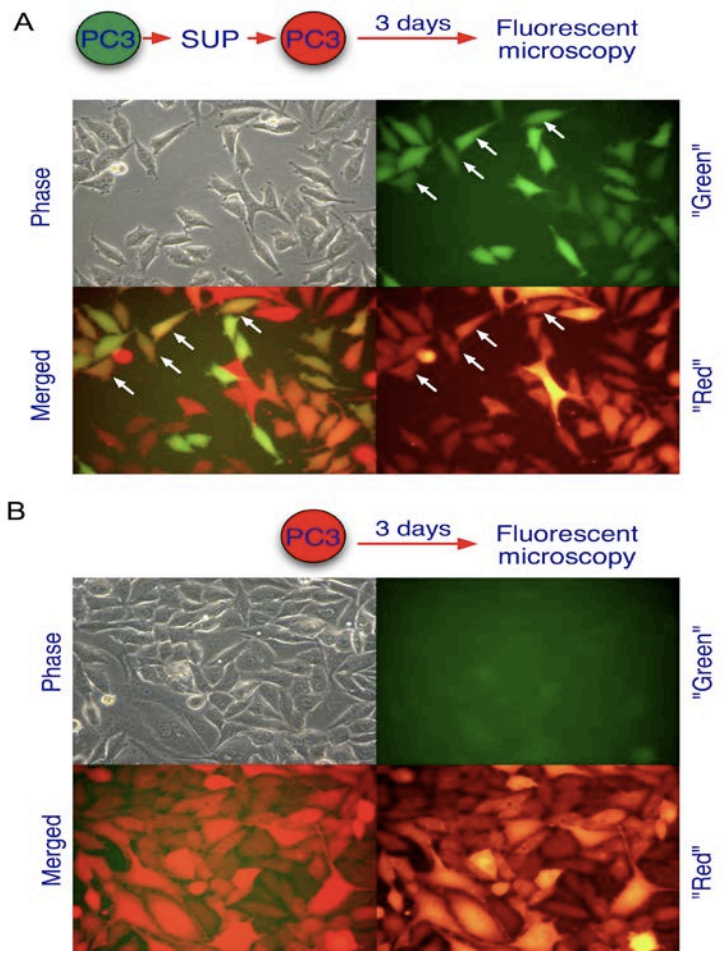


Figure 2. Genetic markers are transferred by a filterable activity. A. Tissue culture medium conditioned by "green" PC3 cells was passed through a .45 μ and added to "red" PC3 cells, which were analyzed by fluorescence microscopy in three days. "Red" PC3 cultured in normal medium were used as a control (B). Some of the cells in which expression of EGFP was induced are indicated by arrows.

Several observations led us to exclude cell fusion from consideration. We found that co-culturing "red" and "green" PC3 cells produced cells that expressed both fluorescent proteins (Fig. 1), thus showing that the effect that was observed by Dr. Glinsky in vivo could be recapitulated in vitro. However, we could not detect cell fusion by time-lapse microscopy, which would be surprising, if it did happen, considering the high incidence of "yellow" cells (Fig. 1). We also failed to detect "yellow" cells that were binuclear or multinuclear, which would be an immediate consequence of cell fusion. Finally, we found that culturing "red" cells in a filtered tissue culture medium conditioned by "green" cells produced "yellow" cells (Fig. 2). This implied that gene transfer did not require direct cell-

cell contact, which also was not consistent with cell fusion.

The finding that gene transfer was mediated by a filterable activity suggested two mechanisms. One, was engulfment of apoptotic bodies. This hypothesis appeared unlikely because the size of the bodies would preclude them from passing through the filter and because the rate of apoptosis in PC3 cells was too low to explain the high incidence of gene transfer. Prompted by our observations from an unrelated study, we considered an alternative explanation, that the *EGFP* and *RFP* genes could be transferred between cells by a virus. Indeed, *EGFP* and *RFP* were transduced into PC3 cells by retroviral vectors. Therefore, if PC3 cells carry a replication competent retrovirus, this retrovirus could transfer *EGFP* or *RFP* by two mechanisms: by recombining with the vectors, or by packaging the RNA expressed by these vectors into infectious particles. For convenience, we named this hypothetical virus PC3V.

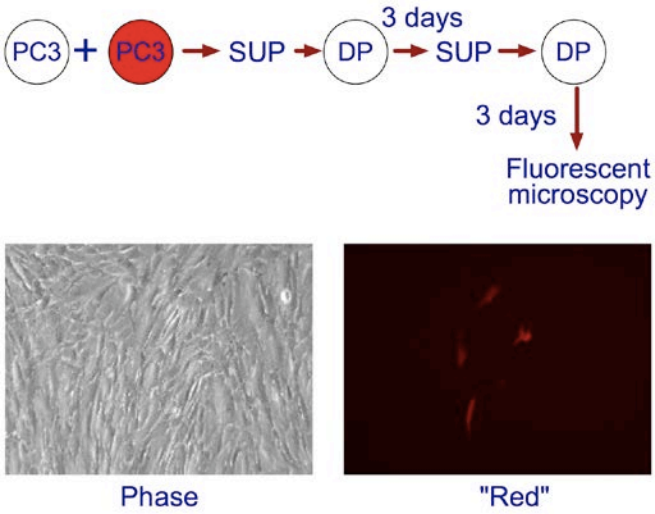


Figure 3. The activity that transfers genetic markers of PC3 cells has properties of a virus. Tissue culture conditioned by "red" PC3 cells was passed through a .45µ filter and added to human fibroblasts that expressed a dominant negative mutant of P53 (DP cells). The cells were washed with fresh unconditioned medium the next day and cultured for three days. The medium conditioned by the cells was applied to naïve DP cells, which were analyzed in three days, which revealed the expression of the RFP gene. This figure was provided by Dr. Glinsky.

The viral transfer hypothesis predicted that the virus from "green" or "red" PC3 cells should be able to propagate in other human cells. We tested this prediction by incubating human fibroblasts with tissue culture medium conditioned by "red" PC3 cells (Fig. 3). The treated fibroblasts indeed began to express RFP, which was consistent with our hypothesis. These results were reproduced and extended by Dr. Glinsky, who found that genes encoding EGFP and RFP could be transferred independently. The model that a virus was surreptitiously transferring the genetic markers raised several questions. What is this virus? Where did it come from? Does it have oncogenic properties? To answer these questions, we set up to identify PC3V.

We considered two possibilities. First, that PC3V recombined with the retroviral vector encoding EGFP or RFP. If true, then identity of the virus could be revealed by infecting original PC3 cells with the virus from "green" PC3 cells and then obtaining the sequence of the DNA adjacent to *EGFP*. Alternatively, PC3V could package the RNA expressed by the vector without recombining with it. In this case, purifying the virus and identifying it by peptide sequencing would be informative. Since we had extensive experience in the latter approach, we decided to identify PC3V by purifying it.

To facilitate the purification, we chose a serum-free and protein-free medium in which PC3 could live for several days and still secrete the virus. Using this medium, we purified the infectious activity and fractionated it by gel electrophoresis (Fig. 4).

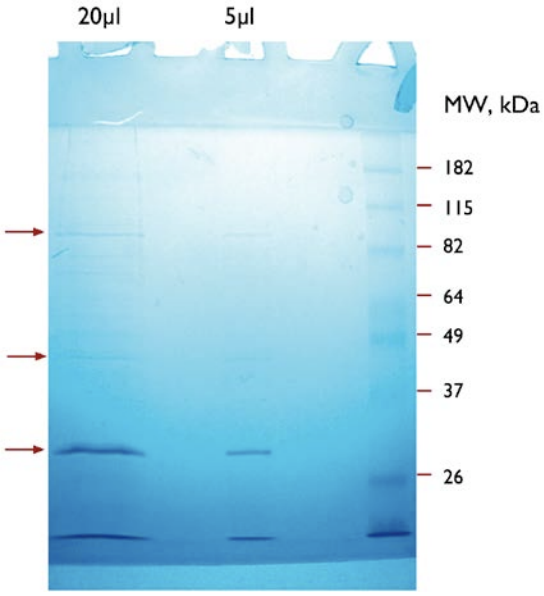
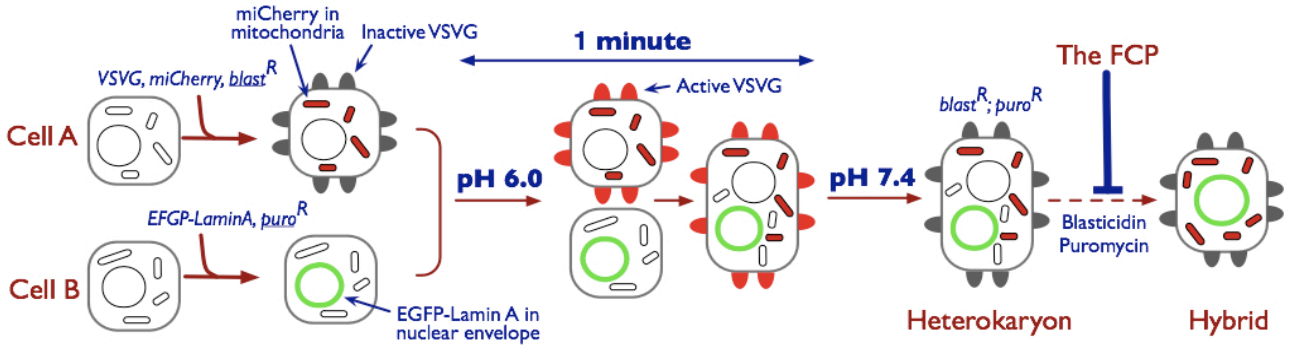


Figure 4. Polypeptides purified from medium conditioned by "green" PC3 cells. "Green" PC3 cells were cultured in the medium containing equal volumes of DMEM and F12 and no serum for three days. The medium was collected, passed through a .45µ filter, clarified by centrifugation at 1000g, the remaining particulate material was pelleted at 100,000g, resuspended in SDS sample buffer, fractionated by electrophoresis (5 µl or 20 µl of the 100 µl sample were loaded) and stained with Coomassie. The polypeptides indicated by the arrows were sent out for sequencing.

This analysis revealed three major polypeptides that were identified by peptide sequencing as fragments of Gag and Env of the mouse leukemia virus (MuLV). The subsequent nucleotide sequencing of the isolated virus yielded about 70% of its sequence, which was 97% to 98% identical to various MuLV or XRMV isolates with the closest homology to DG-75 MuLV, a xenotropic MuLV virus isolated from human lymphoblastoid cells (Raisch et al., 2003). These findings suggested two possibilities.



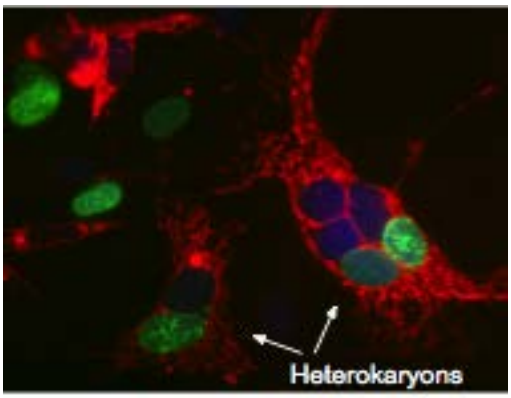


Figure 5. V-fusion. Fusion partners (cells A and B) are transduced with one of two retroviral vectors. One will confer resistance to blasticidin (*blast*^{R}) and carry the gene encoding VSVG, the fusion protein of the vesicular stomatitis virus, and the gene encoding a mitochondria-targeted fluorescent protein Cherry (miCherry). The second confers resistance to puromycin (*puro*^{R}) and carry a gene encoding a fusion between the fluorescent protein EGFP and Lamin A, which localizes to nuclear lamina. The cells are plated together and the medium replaced with PBS at pH 6, which ***reversibly*** activates the fusogenic activity of VSVG, thus initiating fusion of adjacent cells. After one minute of incubation the cells are washed with normal culture medium, which makes VSVG inactive. The heterokaryons can be identified by fluorescence microscopy, as shown, or by phase contrast microscopy.

The simplest explanation, which we favor, was that PC3 cells were infected with MuLV while they were propagated in mice. MuLV are present as complex populations that include xenotropic and polytropic viruses, both of which can infect human cells. Because PC3V did not infect mouse 3T3 cells, but infected human cells, PC3V could be related to xenotropic MuLV. Alternatively, PC3V could be a human virus that had been activated in PC3 cells and was activated after the cells were injected into the mouse. This hypothesis was suggested by the surprising finding (Dong et al., 2007; Urisman et al., 2006) that some human prostate cancers contain a virus, which is closely (about 95% nucleotide identity) related to xenotropic MuLV and, accordingly, was named xenotropic MuLV-related virus (XMRV). At that time, this hypothesis implied that mouse viruses, or viruses closely related to them may have a larger role in human disease than it hag been anticipated. A series of recent studies strongly suggested that finding XMRV in human samples could be explained by contamination of research reagents with MuLV (Cingoz and Coffin, 2011). Before these studies were published, we put our research on PC3V on hold because Dominik Duelli, the postdoctoral fellow who worked on the funded project, left to establish his own laboratory at the Rosalind Franklin University. Because I could not secure an NIH grant, I was not allowed to hire a replacement for Dominik.

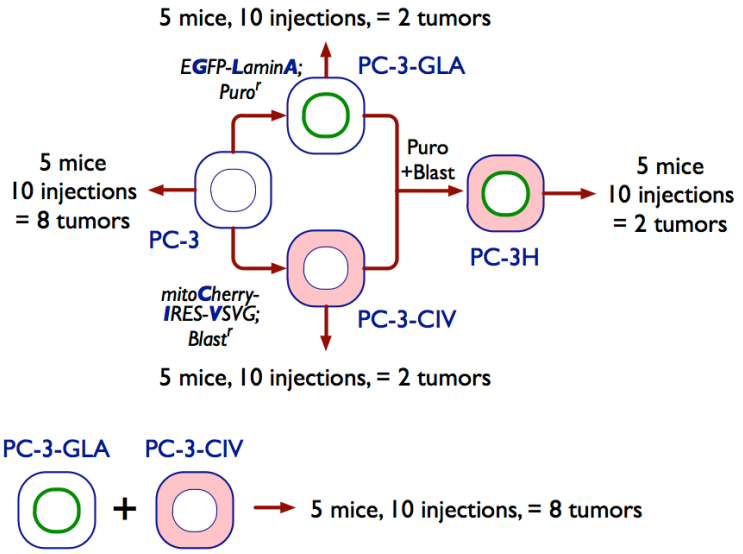


Figure 6. Comparing tumorigenicity of hybrids and the parental cell lines. PC3 cells were processed and injected as indicated.

Accomplishing Aim 1 led to several conclusions. One, that horizontal transfer of genes by retroviruses, and mouse xenotropic viruses in particular, could be an underappreciated factor in experimental models. Indeed, a majority of retroviral vectors are based on mouse viruses, and thus can be packaged by replication competent retroviruses present in the transduced cells if these cells are of mouse origin or were in contact with mouse cells. Therefore, the transgenes can then surreptitiously spread to other cells, thus affecting the interpretation of the experiments. Another concern is that these viruses can infect experimentalists themselves. Considering that transduction of oncogenes by retroviral vectors is commonly used in cancer research, our findings

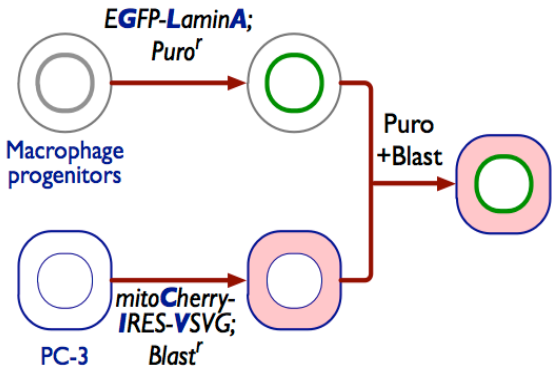


Figure 7. Generation of hybrids between PC-3 and macrophage progenitors.

provide an additional reason to verify that no infectious replicating viruses are generated during such experiments. In particular, human cancer xenografts explanted from rodents can carry viruses capable of transducing human cells with potentially harmful genes.

The outcome of exploring Aim 2: To test whether cell fusion caused by viruses can affect ability of PC3 cells to metastasize.

The main goal of this aim was to test whether fusion of prostate cancer cells among themselves or to normal cells of the host affects the extent or tropism of metastasis. As we quickly found, accomplishing this Aim required solving several technical and conceptual problems that we had not fully appreciated. We needed to develop a new approach to fuse cells, to learn how cell fusion affects cell viability and clonogenic survival, and to consider unexpected in vivo effects of fluorescent markers. Addressing these problems revealed that accomplishing the proposed research required additional resources, people and expertise, which we were unable to secure. However, our findings provided a foundation for future studies.

A new approach to fuse cells. To fuse cells, we originally planned to use MPMV, a primate retrovirus that we had used for this purpose routinely (Duelli and Lazebnik, 2007; Duelli et al., 2005). The finding that using replication competent viruses can complicate interpretation of results (Aim 1), we looked to alternative approaches to cell fusion. The available alternatives included fusion by polyethylene glycol (PEG), which is notoriously toxic, or by inactivated Sendai virus, which is laborious to obtain and expensive to buy. Therefore, we developed a new approach to fuse cells (Fig. 5) (Gottesman et al., 2010). We call this approach V-fusion because it is based on the ability of the vesicular stomatitis virus G protein (VSV-G) to become reversibly activated at pH 6.0 or below. Cells that express VSV-G remain intact under normal tissue culture conditions, fuse if exposed for a minute to a buffer with pH 6.0 or below, and cease to fuse once returned into tissue culture medium. We made a set of retroviral vectors (pIV) that express VSV-G from an internal ribosome entry site (IRES) at an optimal concentration and have a cloning site to introduce genes encoding fluorescent tracers or other proteins of interest. This approach is simple, easily scalable, and versatile, which suggests that it can benefit a variety of studies that use cell fusion as a tool or investigate the consequences of cell fusion itself.

Comparing tumorigenic activity of PC3 cells and its hybrids. We proposed to test whether hybrids of PC3 cells with themselves or with normal cells increase the metastatic potential of this cell line. To this end, we obtained clonogenic hybrids of PC-3 cells using V-fusion (Fig. 6) and injected the resulting hybrids, the parental cell lines (PC-3-GLA and PC-3-CIV), and a mixture of PC-3-GLA and PC-3-CIV into the nude mice. We found that the hybrids produced as many tumors as the parental cell lines, which suggested that fusion among PC3 cells did not result in cells with increased tumorigenicity. However, the parental cells (PC-3-GLA and PC-3-CIV) produced fewer tumors than the original PC-3 cells, which was surprising because the transgenes were not expected to affect tumorigenicity. To find considered alternative ways of labeling cells and selecting the hybrids we designed and tested in vitro retroviral and lentiviral vectors that introduce

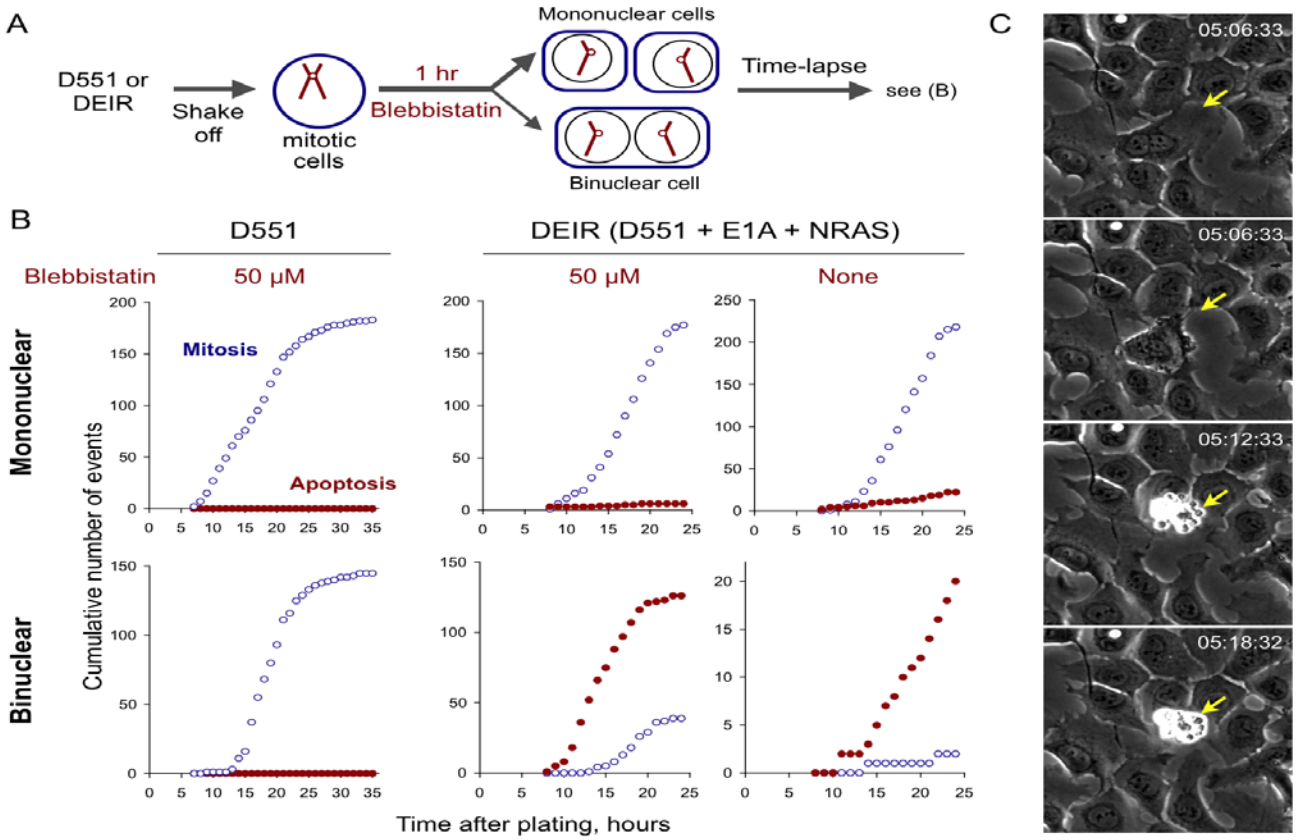


Figure 8. Preventing cytokinesis causes oncogene-dependent apoptosis. A. Experimental design. Mitotic cells were obtained by shake-off from asynchronous cell populations of normal human fibroblasts Detroit 551 (D551) or DEIR cells (D551 transduced with a retroviral vector expressing E1A and oncogenic NRAS). The mitotic cells were plated in medium containing either 50 μM blebbistatin or no drug. The cells were washed one hour later four times to remove the drug and monitored by time lapse microscopy for the incidence of mitosis and apoptosis, which were plotted in **(B)** as a cumulative number of events over the time elapsed after cell plating. An example of a typical even that was scored as apoptosis is presented in C. The time stamps in (C) indicate hours:minutes:seconds from the start of the experiment. The results in (B) are a summary of two independent experiments. DEIR cells were used within 10 passages following retroviral transduction.

EGFP and mCherry fused to drug resistance proteins, histones, and targeted them to membranes. Testing how these tracers affect tumorigenicity of the cells will require additional effort.

One hypothesis that we proposed to test in our application was that fusion of tumor cells to macrophages can enable metastasis of tumor cells. We developed an approach (Fig. 7) to fuse PC-3-CIV cells to mouse macrophage progenitors, which were obtained through our collaboration with Dr. James Bliska (Stony Brook University). The resulting hybrids proliferated well, which demonstrated that such hybrids could be generated. Testing tumorigenic properties of these hybrids will require future studies.

How does cell fusion affect clonogenic survival? Our initial attempts to obtain clonogenic hybrids of PC-3 cells led us realize that the mechanisms that control the fate of fused cells are poorly understood. To learn these mechanisms better, we turned to an experimental system that we used previously to study regulation of cell viability by oncogenes and exploited to understand how cell fusion can contribute to carcinogenesis. In this experimental system, normal diploid human fibroblasts are co-transduced with the adenoviral oncogene *E1A*, which deregulates cell cycle, and an oncogenic mutant of *RAS,* which has multiple effects, including inhibiting apoptosis caused by cell cycle deregulation. The resulting cells have epithelioid morphology, express markers of epithelial cells, proliferate in soft agar and, if provided with an additional oncogene, make tumors in nude mice. The use of normal diploid fibroblasts as the initial material and the use of defined oncogenes provided the benefit of using relatively well defined and homogeneous population of cells, which facilitated interpretation of the results.

Our hypothesis was that the viability and clonogenic survival of heterokaryons and cell hybrids could be affected by tetraploidy, a condition that is a natural consequence of cell fusion and which had been associated with cell cycle arrest and apoptosis (Ganem et al., 2007). Therefore, we compared effects of cell fusion and cytokinesis failure, as both of these processes cause tetraploidy. To prevent cytokinesis, we used an established approach (Fig. 8A), in which mitotic cells obtained from an asynchronous population were incubated with blebbistatin at a concentration low enough to make only some cells binuclear, while others were able to complete cytokinesis, producing mononuclear cells. Thus, we were able to compare the fate of cells that were treated identically in the same dish, but became either binuclear (tetraploid) or mononuclear (diploid). We used time-lapse microscopy to score mitosis, apoptosis, and cell cycle arrest. The majority of binuclear fibroblasts (Fig 8, D551) completed the first cell cycle and mitosis, consistently with the reports that tetraploidy does not cause cell cycle arrest. However, binuclear D551 transduced with *E1A* and *NRAS* (DEIR cells) died by apoptosis (Fig. 8B) as indicated by changes in morphology and the ability of the apoptosis inhibitor Bcl-2 to completely prevent cell death. Apoptosis was caused by cytokinesis failure and not by side effects of blebbistatin, because mononuclear cells remained viable (Fig 8B) and binuclear cells that were formed occasionally in the absence of blebbistatin (Fig. 8B, right column) also underwent apoptosis.

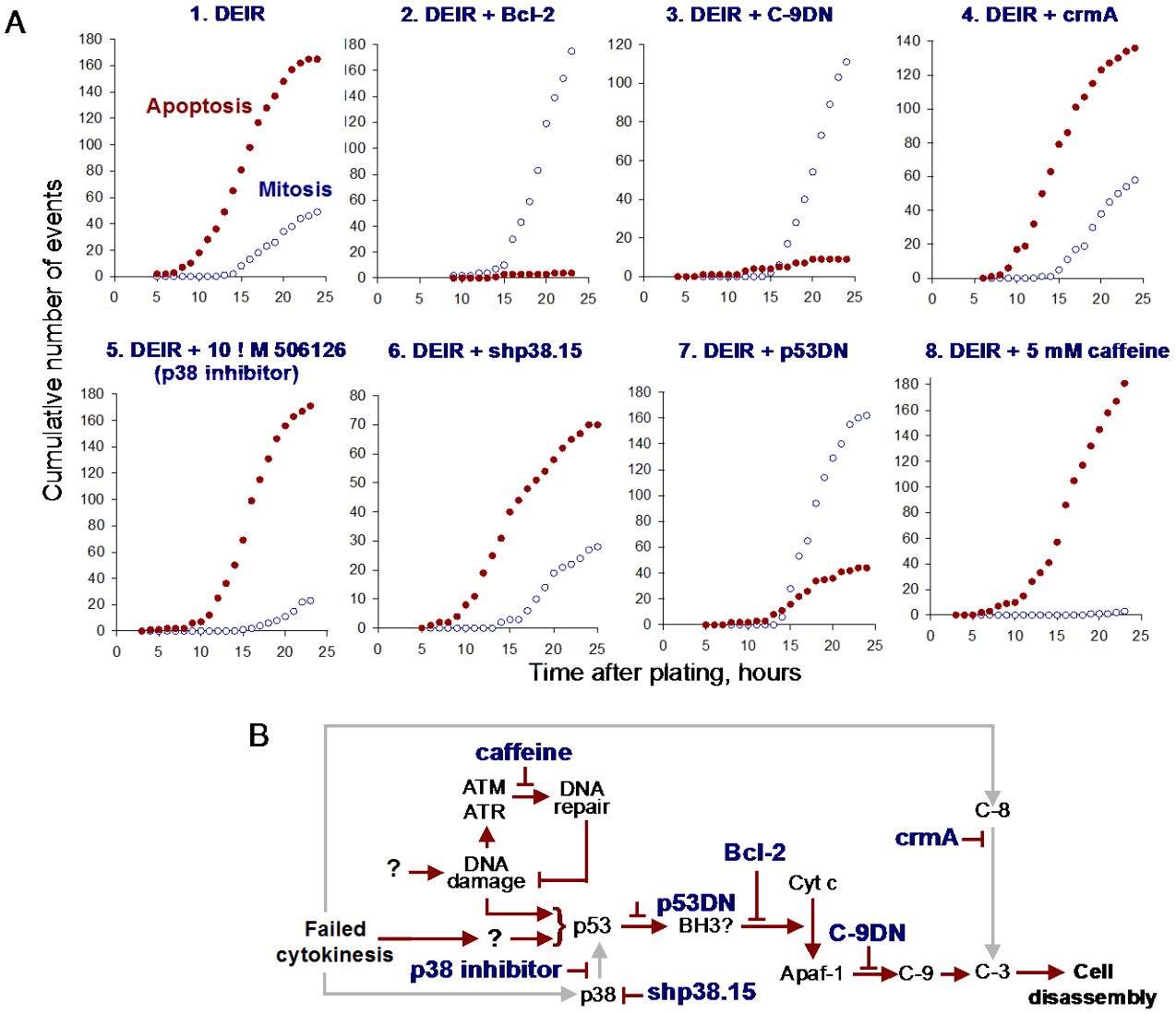


Figure 9. Failed cytokinesis activates the intrinsic pathway of apoptosis. (A) DEIR cells (A.1, A.5, A.8), DEIR cells expressing the indicated genes (A.2, A.3, A4, A.7), or an shRNA to p38 (A.6) were treated with blebbistatin as in Fig. 1A to inhibit cytokinesis and then monitored by time-lapse microscopy to record the incidence of mitosis (blue open circles) and apoptosis (red filled circles) in binuclear cells. A p38 inhibitor (A.5) or caffeine (A.8) were added together with blebbistatin and then present in the medium throughout the experiment. **(B)** A summary and interpretation of the experiments presented in A. The red arrows represent the pathways that are consistent with the results in A, the grey arrows represent the pathways that are not. The incidence of apoptosis in mononuclear cells was not increased by any of the treatments except caffeine, which increased the incidence of apoptosis slightly. Each graph is a summary of two independent experiments with the exception of A.6, which is the result of one experiment.

To learn how apoptosis caused by cytokinesis failure was regulated, we used a set of apoptosis inhibitors. Apoptosis was prevented either by expressing Bcl-2 (Fig 9A, compare A.1 and A.2), which inhibits the release of cytochrome c from mitochondria, or by expressing a dominant negative mutant of caspase-9

(C9DN) (Fig 9A.3). In contrast, expression of crmA, which inactivates the extrinsic pathway by inhibiting caspase-8, had no detectable effect (Fig 9A.4). To determine if apoptosis is mediated by p53, we expressed a dominant negative mutant of this protein (p53R175H, p53DN), which inhibited apoptosis (Fig. 9A.7). Overall, our observations indicated that cytokinesis failure induced the intrinsic pathway of apoptosis and that the signal transduction events proximal to tetraploidy have yet to be identified.

To learn how cell fusion affects cell viability, we used V-fusion to fuse DEIR cells (Fig. 10), which resulted in a population consisting of binuclear, multinuclear and mononuclear cells. The mononuclear cells continued to proliferate as indicated by the near constant rate of entry into mitosis (Fig. 10B, top row). The time courses of mitosis or apoptosis in control cells, which were treated with regular PBS, were practically identical to that of cells treated with PBS at pH 6.0 (Fig. 3B, top left), indicating that the procedure was not toxic or cytostatic by itself. However, a majority of heterokaryons died by apoptosis within 20 hours following cell fusion, while the rest progressed through cell cycle to complete mitosis (Fig. 10B, bottom left) with only some remaining in interphase by the end of the observation. Similar to mononuclear cells, the nuclei of heterokaryons underwent nuclear breakdown before the cells began to round up during mitosis. Apoptosis induced by fusing cells, as apoptosis induced by inhibiting cytokinesis, was prevented by expressing Bcl-2 (Fig. 10B, DEIR-BIV cells), indicating that the intrinsic pathway was involved. We reasoned that apoptosis could be induced by a common consequence of cell fusion and cytokinesis failure, or cell fusion could also induce apoptosis by an unrelated mechanism, perhaps triggered by fusing asynchronous cells.

To test this possibility, we fused cells shortly after they exited mitosis, which imitated cytokinesis failure and that the nuclei in the resulting cells were still synchronous (Fig. 11). The time courses of mitosis (Fig. 11B) and apoptosis (Fig. 11C) were similar for cells that were obtained either by inhibiting cytokinesis or by “reversing” it through cell fusion following cell division. These observations were consistent with the model that cell fusion and cytokinesis failure induce apoptosis through a common mechanism, which could be triggered by tetraploidy, the presence of two nuclei, or by other common consequences. However, apoptosis in heterokaryons that were produced by fusing asynchronous cells (asynchronous heterokaryons) occurred noticeably earlier than in heterokaryons obtained by “reversing” cytokinesis (Fig. 11C), and happened more often during mitosis that during the interphase (Fig. 11D,E). The prevalence of mitotic apoptosis in asynchronous heterokaryons (Fig. 11E) suggested that this death could be caused by cell cycle asynchrony of the nuclei. Determining how this asynchrony causes apoptosis will require closer examination. Overall, we concluded that cell fusion causes cell death by at least two mechanisms, one of which is common with that triggered by cytokinesis failure.

Preventing apoptosis by expressing Bcl-2 did not increase clonogenic survival of tetraploid cells produced by either cytokinesis failure or cell fusion, while expressing a dominant negative mutant of the tumor suppressor p53 did (Fig. 12). These findings implied that tetraploidy induces more than one process that prevents clonogenic proliferation of these cells, a conclusion consistent with the reports that tetraploidy causes cell cycle arrest. Our findings were also inconsistent with our previous studies in which we were

able to produce clonogenic hybrids by fusing cells that expressed E1A or HRAS. Some differences between the old and the new protocols that we used suggested two explanations. One was that clonogenic survival depended on MPMV, the virus that we used previously to fuse cells. Another possibility was that previously, we transduced E1A and HRAS separately, and then fused the resulting cells, while in the current experiments we co-transduced the oncogenes and then fused the resulting cells. Using V-fusion instead of MPMV produced clonogenic hybrids (Fig. 12), suggesting that cell fusion and cytokinesis failure have different potential to produce clonogenic tetraploid cells. The experimental system that we have developed might help to understand the mechanisms underlying this difference.

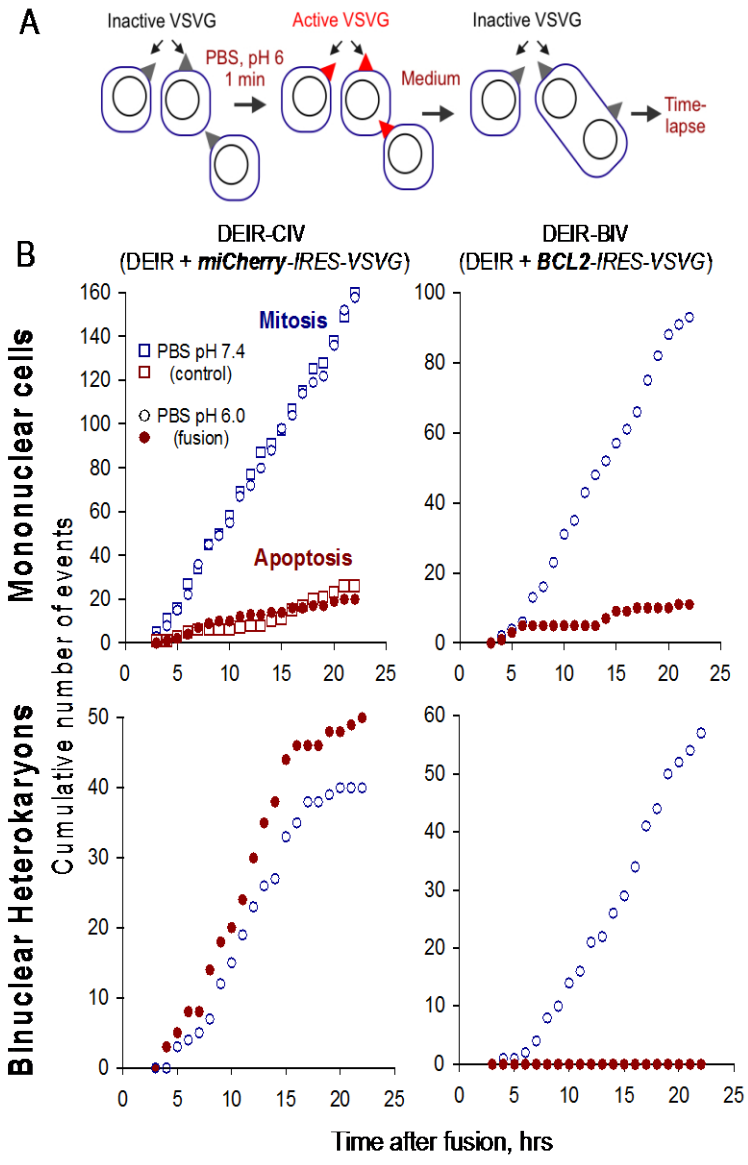


Figure 10. Cell fusion induces apoptosis. (A): Cell fusion approach. Cells of interest are transduced with one of the vectors indicated in (B), which express VSVG, a viral fusogen that is activated by incubating the cells at pH 6.0 for one minute. The resulting heterokaryons (cells containing two or more nuclei) are detectable by phase contrast microscopy within 15 minutes. **(B)**. D551 transduced with a vector expressing miCherry and IRES-driven VSVG (DEIR-CIV cells) or a vector expressing BCl-2 and IRES-driven VSVG (DEIR-BIV cells) were fused as in A. The remaining mononuclear cells (top panels) and binuclear heterokaryons (bottom panels) were monitored by time-lapse microscopy (Materials and Methods) to record the incidence of mitosis and apoptosis. The rates of mitosis and apoptosis in cells that were treated with normal PBS are presented in top left graph (pH 7.4, control). All results are from two independent experiments.

The following study was completed under the no cost extension (NCE).

An oncogene causes multipolar mitosis. While studying the effects of cell fusion and cytokinesis failure on cell viability, we unexpectedly noticed that binuclear and multinuclear cells produced either by cytokinesis

failure or cell fusion underwent multipolar mitosis much more often that mononuclear cells. Furthermore, we found that this effect depended on expression of E1A (Fig. 13). Considering the high incidence of multipolar mitosis, the ability of multipolar mitosis to cause chromosomal aberrations, that *E1A* belongs to a common human virus, and that this oncogene has been used for cancer treatment, we investigated how E1A affected mitotic polarity and analyzed the fate of the resulting progeny.

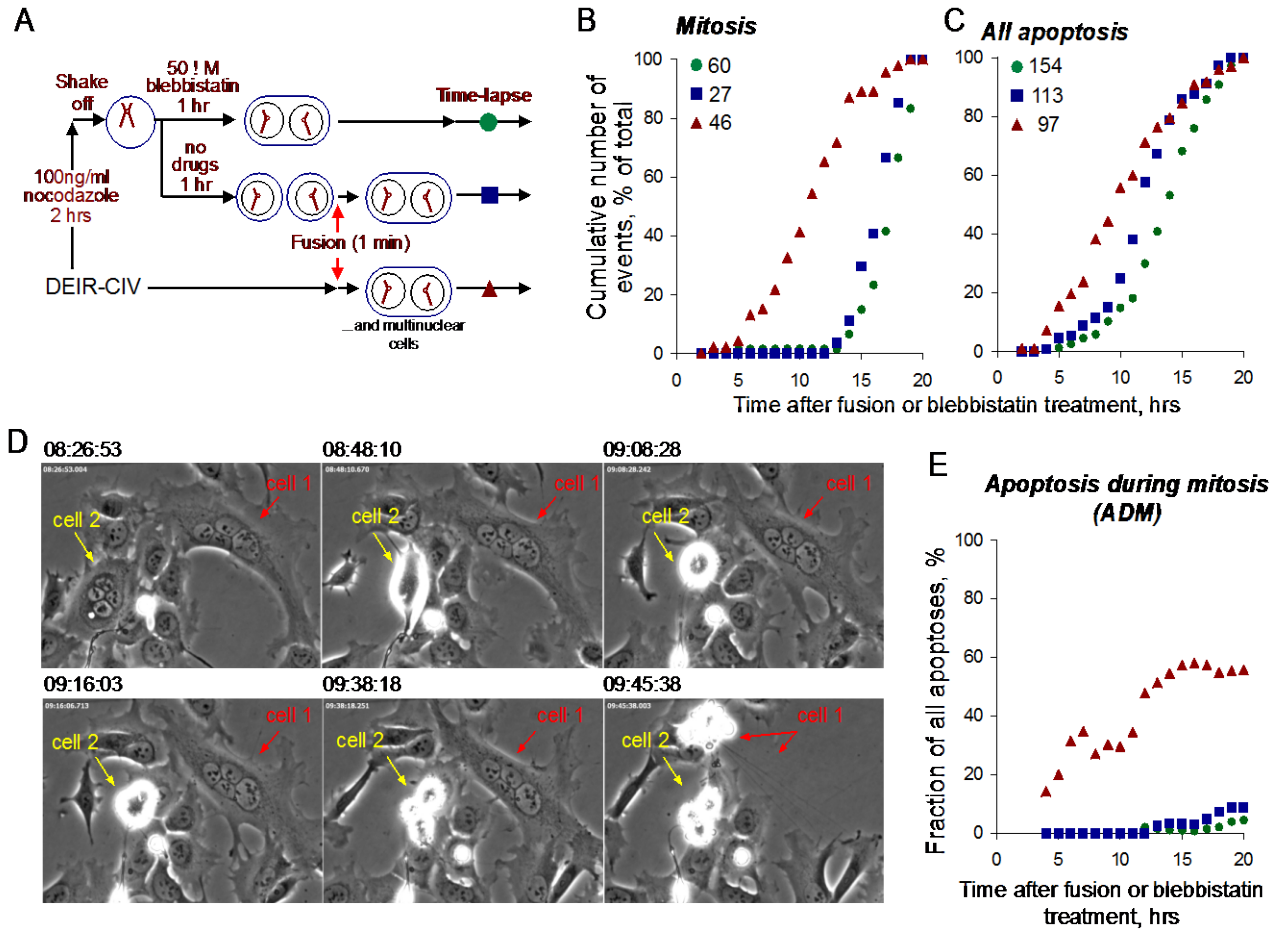


Figure 11. Asynchronous cell fusion causes apoptosis during mitosis. (A) Flow chart of the experiment to test whether fusing synchronized cells also induces apoptosis. We compared the fate of binuclear DEIR-CIV cells that were obtained by inhibiting cytokinesis (top protocol), to heterokaryons obtained by fusing cells shortly after they complete cytokinesis ("reversed" cytokinesis, middle protocol), and heterokaryons obtained by fusing asynchronous cells (asynchronous heterokaryons, bottom protocol). The cells were monitored by time-lapse microscopy to record the incidence of mitosis **(B)** and apoptosis **(C)**. The symbols in the graphs correspond to the symbols used to label each protocol in A. To facilitate the comparisons, we expressed the results as a fraction of the total number of observed events. The absolute number of events is indicated in the top left corner of each graph. Note that the time course of apoptosis in heterokaryons with two nuclei was superimposable to that of heterokaryons with three or more nuclei (Supplemental Figure 3). **(D)** An example of ***a***poptosis ***d***uring ***i***nterphase (ADI) (cell 1) and ***a***poptosis ***d***uring ***m***itosis (ADM) (cell 2) occurring in asynchronous heterokaryons. The time stamp in hours:minutes:seconds indicates the time from the beginning of the recording. **(E)** The incidence of ADM as a fraction of the total incidence of apoptosis. Each graph is a summary of two independent experiments.

E1A functions as an oncoprotein by directly deregulating several tumor suppressors (Fig. 14A). The primary targets are the RB family, which regulates cell cycle, apoptosis, and cellular senescence, and chromatin remodelers p300 and CBP (p300/CBP), which change expression of numerous genes in response to a variety of signals. To test whether E1A caused multipolar mitosis by deregulating either of these families, we tested previously characterized mutants that fail to bind and thus deregulate either RB (E1AΔCR2) or p300/CBP (E1AΔ2-11). E1AΔCR2 induced multipolar mitoses as efficiently as E1A, while E1AΔ2-11 had no detectable effect (Fig. 14B), suggesting that E1A affected mitotic polarity by deregulating p300/CBP.

To learn how deregulation of p300/CBP caused multipolar mitosis, we considered that multipolar mitosis requires a combination of two events: i) attaining extra centrosomes, which organize extra poles of the mitotic spindle and can be acquired through cell fusion, cytokinesis failure, or abnormal amplification; and ii) inhibiting poorly understood mechanisms that force bipolar mitosis even in the presence of extra centrosomes by inactivating some of them or by clustering all centrosomes into two groups. Because E1A caused multipolar mitosis only in cells made by cell fusion or cytokinesis failure (Fig. 14) but not in mononuclear cells, and because we found no evidence that expression of E1A caused abnormal centrosome amplification, we reasoned that E1A inhibited centrosome clustering or inactivation.

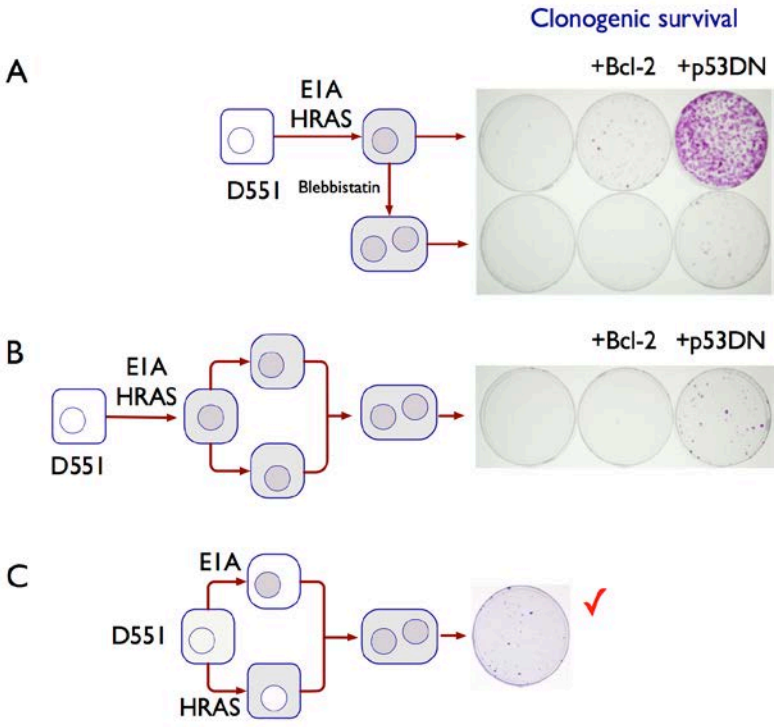


Figure 12. Distinct consequences of cell fusion and cytokinesis failure for clonogenic survival. D551 – normal diploid human fibroblasts Detroit 551.

To visualize centrosome clustering, we transduced DERP cells (DEIR cells that expressed $p53^{R175H}$) with two fluorescent proteins: γ-tubulin-EGFP, a marker of centrosomes, and histone 2A-Cherry (H2A-Cherry), a

marker of chromatin. We then made the resulting cells tetraploid by inhibiting cytokinesis and visualized both markers by confocal time-lapse fluorescent microscopy while the cells were proceeding through mitosis (Fig. 15A). At the onset of mitosis, the four centrosomes present in binuclear cells separated to associate with the poles of a tetrapolar mitotic figure (Fig, 15B). Mitosis then progressed along one of three routes (Fig. 15B, R1 – R3). In one (Fig. 15B, R2), the centrosomes clustered into two groups, resulting in a bipolar mitosis. Alternatively, four centrosomes remained separated (Fig. 15B, R1), leading to a mitosis that produced either four (Fig. 15B, R1.1), or three (Fig. 15B, R1.2) daughters, in which case two budding daughters reunited into a binuclear cell. In a third scenario, two out of four centrosomes clustered resulting in mitosis that produced either three (Fig. 15B, R3.1) or, if two budding daughters united into a binuclear cell, two cells (Fig. 15B, R3.2). Because centrosome clustering correlated with the number of daughter cells, we concluded that E1A caused multipolar mitosis by inhibiting centrosome clustering.

An intuitive and thus commonly shared view is that a tetraploid precursor can yield cancers whose ploidy ranges from triploid to tetraploid, while diploid or near-diploid cancers arise without a tetraploid intermediate. Unexpectedly, we found that as much as a third (114 out of 311) of all mononuclear daughters detected while analyzing centrosome clustering resulted from tetrapolar mitoses (Fig. 15B, R1.1, R1.2). Assuming equal chromatin distribution among the daughters, our observation meant that multipolar mitosis could produce near-diploid cells with substantial frequency. Taken together, these observations implied that some diploid or near diploid cancers could descend from a tetraploid precursor, and thus the role of tetraploidy in carcinogenesis might be larger than previously expected.

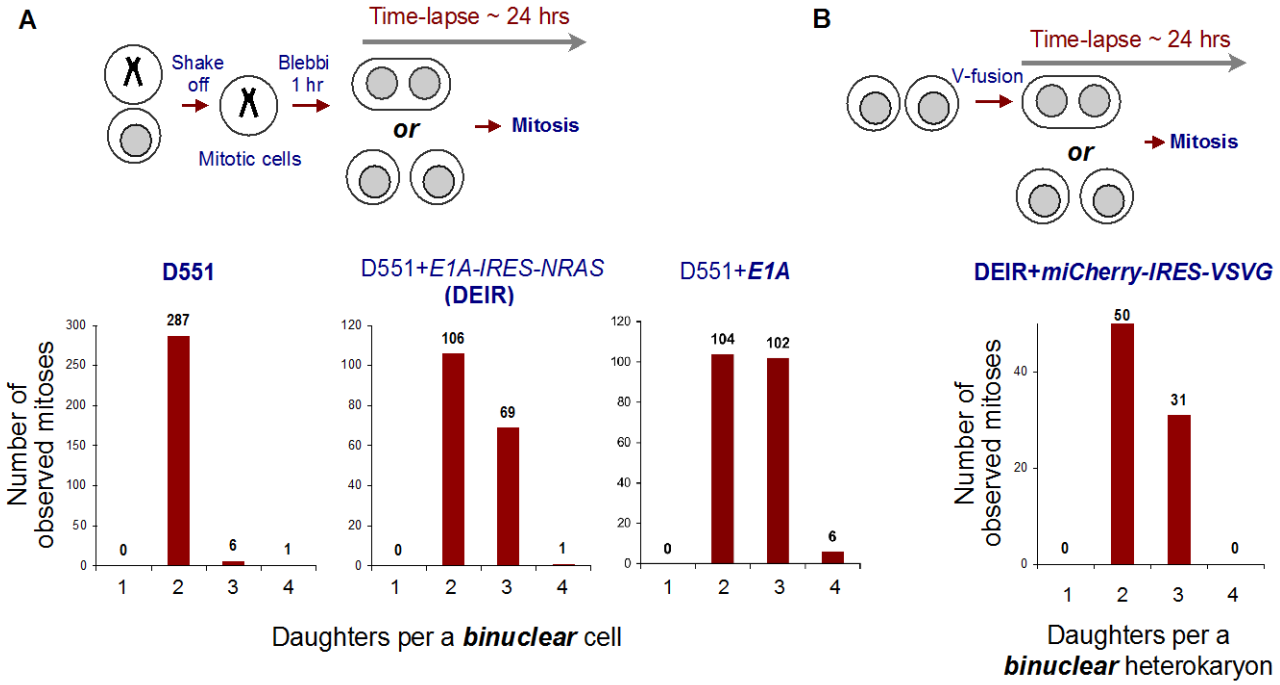


Figure 13. Oncoprotein E1A causes multipolar mitosis. (A) E1A causes multipolar mitosis in cells that bypassed cytokinesis. The indicated cell lines were subjected to mitotic shake-off and the obtained cells were plated into a six-well plate for one hour in medium containing 50 μM (DEIR) or 100 μM (D551) of (±) blebbistatin, which inhibited cytokinesis in a fifth to a half of the population, resulting in binuclear cells, while the remaining mitotic cells divided normally, producing mononuclear cells. After blebbistatin was washed away the cells were recorded by time-lapse microscopy (Supplemental Movie 1 is a sample of the recordings) until the majority of the binuclear cells divided (~24 hrs) to score the number of daughter cells for binuclear (this figure) and mononuclear (Supplemental Fig. 1) cells. (B) Binuclear heterokaryons expressing E1A undergo multipolar mitosis. DEIR cells were fused using V-fusion (Gottesman et al., 2010) and the resulting heterokaryons as well as the cells that remained mononuclear were monitored by time-lapse microscopy for 20 - 24 hours to record the number of daughter cells produced during their first mitosis. For the sake of fair comparison to the cells that bypassed cytokinesis only binuclear heterokaryons were scored. The graphs represent combined data from two (A, D551+E1A), four (B), five (A, D551), or ten (A, DEIR) independent experiments.

The argument that multipolar mitosis can contribute to carcinogenesis would be of little practical significance if the resulting cells were as unviable as it has been widely, although not universally held. To determine how viable such cells are in our experimental system, we monitored the fate of progeny derived from bipolar or tripolar mitoses for several cell cycles (Fig. 15C,D). We found that death was indeed more frequent among the descendants of tripolar mitoses and the incidence of mitosis among them was lower (Fig. 15D, table), but overall, the progeny of tripolar mitoses was far from being moribund (Fig. 15D, graph). To verify this conclusion we repeated the experiment, but instead of tracking the cells by time lapse-microscopy, we scored their chromosome numbers three days after making them binuclear. The resulting population included a range of cells from diploid to tetraploid with a prominent subpopulation in the triploid range, a distribution consistent with the expected ploidy of cells derived from both bipolar and multipolar mitoses. In a separate experiment, we let the cells grow as clones for a month before scoring their chromosome number. The ploidy of the clones was consistent with the conclusion that some of them were progeny of multipolar mitoses, although the design of the experiment did not allow us to make this link unambiguously. Overall, we concluded that progeny of multipolar mitosis could be sufficiently viable to be considered as contributors to carcinogenesis. The view that multipolar mitosis is moribund is based on the notion that multipolar mitosis is "chaotic", in that chromosomes distribute among multiple daughter cells randomly as individual entities, thereby causing frequent nullisomy and consequent death. To test whether this distribution is indeed chaotic, we made heterokaryons in which chromatin of one nucleus was labeled with histone H3.1 linked to EGFP ("green" chromatin), while chromatin of the other with H3.1 linked to mCherry ("red" chromatin) (Fig. 16A). Because deposition of H3.1 into chromatin occurs primarily during S-phase, we expected that if "red" and "green" cells happen to fuse after completing their S-phase, the chromatin in each nucleus in the heterokaryon would remain either "green" or "red" from the time of fusion through mitosis.

Indeed, we were able to unambiguously distinguish parental "red" and "green" chromatin during interphase (Fig. 16B), mitosis (Fig. 16C-E), and even in the resulting progeny (Fig. 16F). We expected that if chromosomes were to distribute during mitosis chaotically, then mitotic figures in heterokaryons would be a homogeneous mixture of "green" and "red" chromosomes. However, in both bipolar (Fig. 16D) and multipolar (Fig. 16C,E) mitotic figures parental chromosomes were in large, easily identifiable clusters. These clusters remained segregated even in the daughter cells, as manifested by "green" or "red" areas of their nuclei (Fig. 16F).

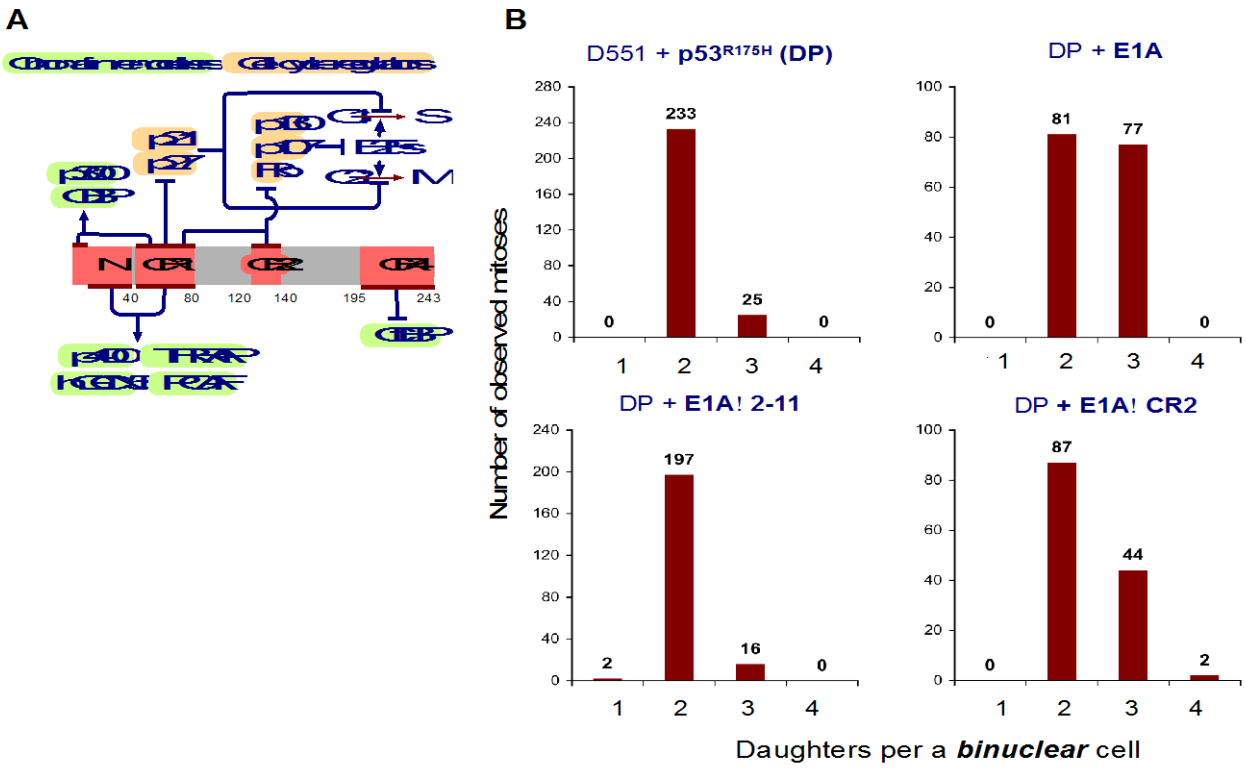
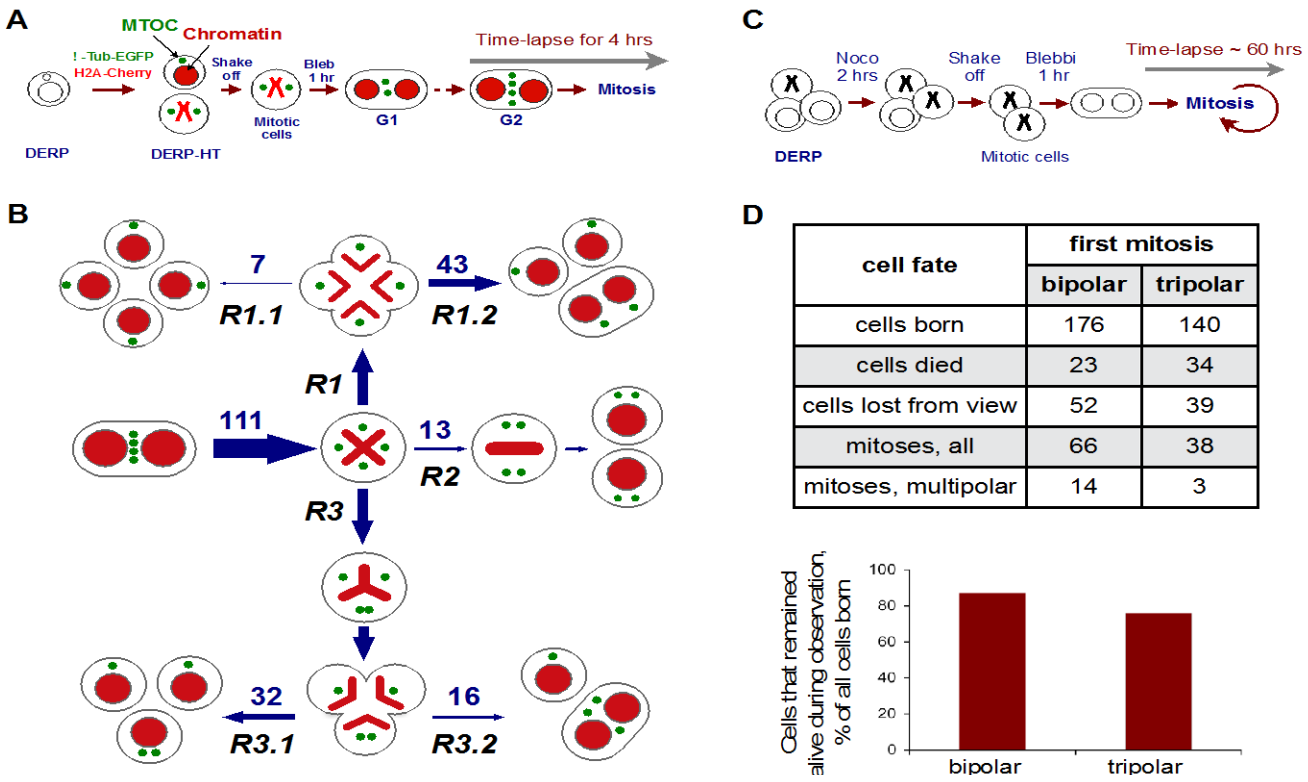


Figure 14. Multipolar mitosis requires the ability of E1A to interact with tumor suppressors p300/CBP. (A) E1A reprograms the target cell by binding and deregulating a set of proteins that control cell cycle or regulate gene expression (adapted from(Gallimore and Turnell, 2001)). (B) E1A mutant that fails to interact with p300/CBP fails to induce multipolar mitosis. D551 fibroblasts were transduced with $p53^{R175H}$ and 72 hours later transduced with E1A, E1AΔCR2, which fails to bind Rb, E1AΔ2-11, which fails to bind p300/CBP, or left untransduced. Forty-eight hours later, the resulting cell lines were processed and recorded to score mitoses of binuclear cells for the number of daughters as described in Fig. 1A. The expression of E1A and the mutants was verified by immunoblotting (Supplemental Figure 2). The graphs are combined data from two independent experiments.

We did observe admixed chromosomes (Fig. 16D, see “red” dots in “green” chromatin) and chromosome clusters of various size (Fig. 4E), which could be predicted to cause numerical aberrations ranging from single chromosome losses and gains to more complex patterns, and thus account for heterogeneity of the resulting progeny. However, overall chromosome distribution during multipolar mitosis did not appear as a random mixture of individual chromosomes.



cell fate	first mitosis	
	bipolar	tripolar
cells born	176	140
cells died	23	34
cells lost from view	52	39
mitoses, all	66	38
mitoses, multipolar	14	3

Figure 15. Multipolar mitoses caused by E1A produce diverse and viable progeny. (A) To visualize centrosomes and chromatin, DERP cells (DEIR transduced with $p53^{R175H}$) were transduced with γ–tubulin-EGFP and H2A-Cherry. The resulting cell line (DERP-HT) was processed with blebbistatin to obtain binuclear cells, which were recorded by confocal time-lapse microscopy as they were entering mitosis. The recordings (Supplemental Movie 2 and 3 are "flattened" fragments of the recordings) were analyzed to determine how the cells divided, which is summarized in (B). The thickness of the arrows in (B) is proportional to the number of cells (numbers in blue) that took a particular route, which were designated as R1.1 to R3.2. Note that expressing γ-tubulin-EGFP increased the incidence of multipolar mitoses (Supplemental Fig. 4), perhaps by interfering with the endogenous γ-tubulin. (C,D) Multipolar mitoses produce viable progeny. DERP cells were processed as shown in (C) to produce a population in which more than 95% were binuclear (Supplemental Fig. 6B). The cells were plated into a six-well plate and recorded for 60 hours starting eight hours after plating. The recordings were analyzed to derive generational trees of the first nineteen recorded bipolar and the first twenty-one recorded tripolar mitoses (Supplemental Fig. 6). The results are presented in (D) as a table of raw data and a graph that presents the fraction of cells that died during observation. 100% in the graph are equal to the total number of cells born (first line of the table). The data in (B) are combined results of three independent experiments; the data in (D) are from one experiment.

These findings support a hypothesis that distribution of chromosome complements among daughter cells, especially during multipolar mitosis, is a regulated process. For example, in normal cells chromosomes could distribute as entire complements, thus yielding euploid progeny, while in neoplastic cells this distribution could be corrupted, thus resulting in diverse, grossly aneuploid progeny. The viability of the progeny would vary accordingly, with euploid cells expected to be more viable than some of the "randomized" cells. Therefore, understanding mechanisms that determine whether chromosomes distribute in complements, clusters, or as individual entities might help to identify new oncogenic pathways that affect genome stability of tetraploid cells. The simple approach that we developed to directly visualize two or more chromosome complements in heterokaryons might help these studies by simplifying interpretation of the data.

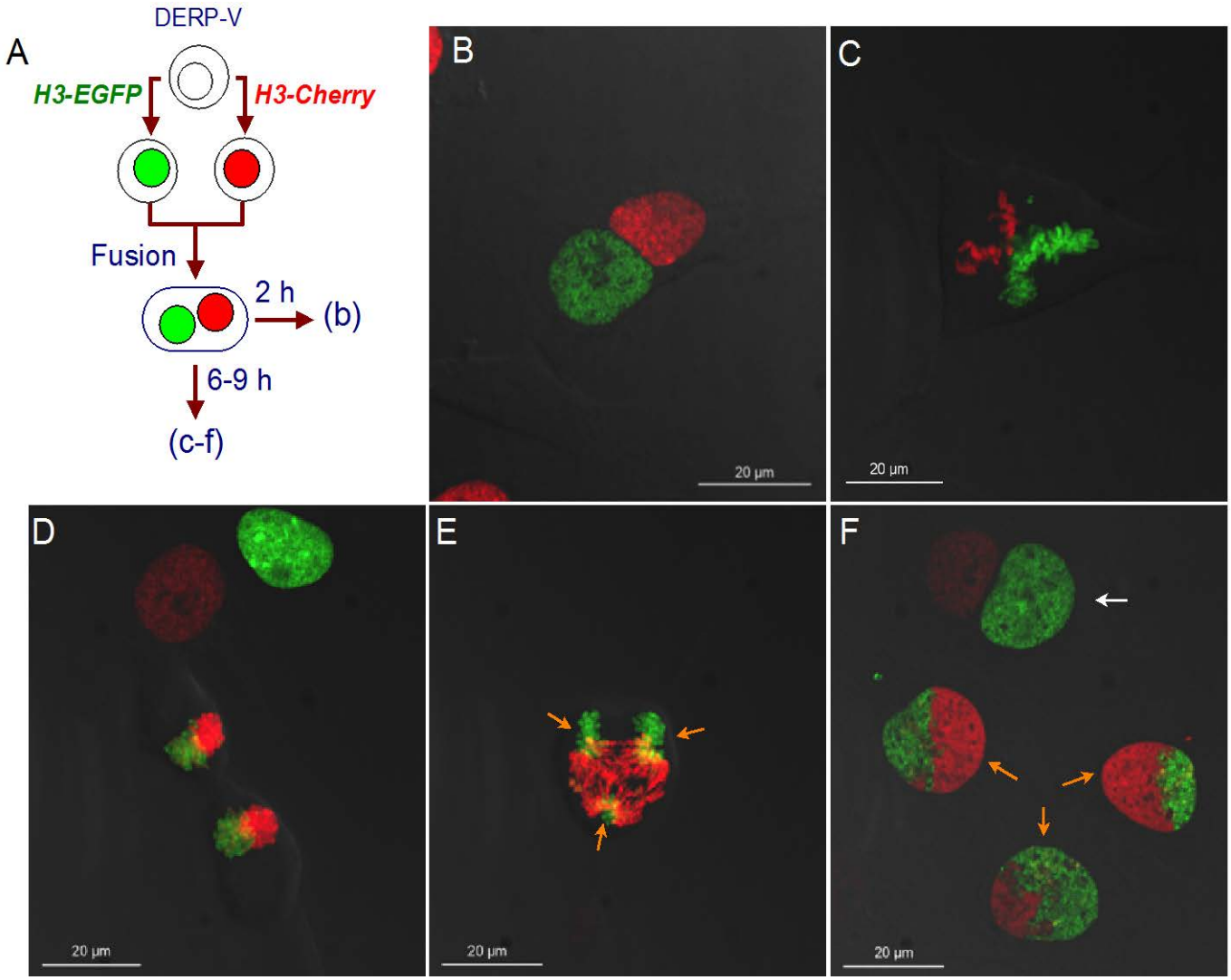


Figure 16. Chromosomes distribute among daughter cells in clusters. (A) To visualize chromatin, DERP-V (DEIR transduced with $p53^{R175H}$ and VSV-G) cells were transduced with either H3.1-EGFP or H3.1-Cherry and then fused by incubating for 1 min. in PBS, pH 6.0. At the indicated time (A) the resulting heterokaryons (B), mitotic figures (C,D,E) and daughter cells (F, indicated by orange arrows) were visualized using confocal fluorescence microscopy. Note typically abutted nuclei in heterokaryons (B), and (F, indicated by a white arrow). The data are from two independent experiments.

KEY RESEARCH ACCOMPLISHMENTS

- A xenotropic replcation competent MuLV virus transfers transgenes between explanted PC3 cells.
- Cell fusion induces apoptosis by a mechanism different from tetraploidy.
- Adenoviral oncogene E1A causes multipolar mitosis in tetaploid cells by inhibiting centrosome clustering.
- Chromosome distribution of parental cells following cell fusion is not random.

REPORTABLE OUTCOMES

Publications:

Duelli, D., and Y. Lazebnik. 2007. Cell-to-cell fusion as a link between viruses and cancer. *Nat Rev Cancer*. 7:968-976.

Duelli, D.M., H.M. Padilla-Nash, D. Berman, K.M. Murphy, T. Ried, and Y. Lazebnik. 2007. A virus causes cancer by inducing massive chromosomal instability through cell fusion. *Curr Biol*. 17:431-437.

Gottesman, A., J. Milazzo, and Y. Lazebnik. 2010. V-fusion: a convenient, nontoxic method for cell fusion. *Biotechniques*. 49:747-750.

Lazebnik, Y. 2010. What are the hallmarks of cancer? *Nat Rev Cancer*. 10:232-233.

Presentations in which I presented the research funded by this grant:

2007

MRC Toxicology Unit, Leicester, UK
The Beatson Institute, Glasgow
University of Zurich
Molecular life science (MLS) graduate school, Zurich
Cell fusion, Lateral Oligonucleotide transport and Cancer, Söderköping, Sweden

Karolinska University, Sweden
Fox Chase Cancer Center
Gordon Conference on Cell-Cell Fusion
Temple University

2008

2nd Conference on Aneuploidy and Cancer
Albert Einstein College of Medicine
National Institute of Child Health and Human Development, NIH
University of Oklahoma
University of Virginia

2009

University of Texas Southwestern Medical Center
Brookhaven National Laboratory
Rosalind Franklin University, Chicago
The City University of New York
Spemann Graduate School of Biology and Medicine, University of Freiburg, Germany
South Illinois University Medical School
Progenitor cells, Micro-environment and Cell Fusion in Cancer Progression, Stockholm
Stony Brook University, Department of Pathology.
Progenitor Cells, Micro-environment and Cell Fusion in Cancer Progression. Stockholm, Sweden

2010

ERASysBio Summer School on Data Management for Systems Biology, Tenerife, Spain
Fordham University, New York, NY
University of Bergen, Norway
NCI Cancer Biology Branch Workshop on Cell Fusion and Exosomes, Bethesda.

2011

Gordon Conference on Cell Fusion
Johns Hopkins University
University of Vermont

CONCLUSIONS

Overall, we accomplished Aim 1 and partially accomplished Aim 2. Both lines of research substantially expanded our understanding of mechanisms underlying horizontal gene transfer and created a foundation for definitive testing of the primary hypothesis - that cell fusion contributes to metastasis. During the funded period, we also provided the scientific community with the review of evidence that underlies the proposed research, with the emphasis on the role of fusogenic viruses in carcinogenesis. The need to evaluate the proposed hypotheses in the context of the prevailing views on the origin of cancer also prompted us to look more critically on these views, and on the concept of the hallmarks of cancer in particular.

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